Studies on Ligand Directed Enzyme Prodrug Therapy and Production of Long Acting Protein Therapeutics for Targeted Cancer Treatment

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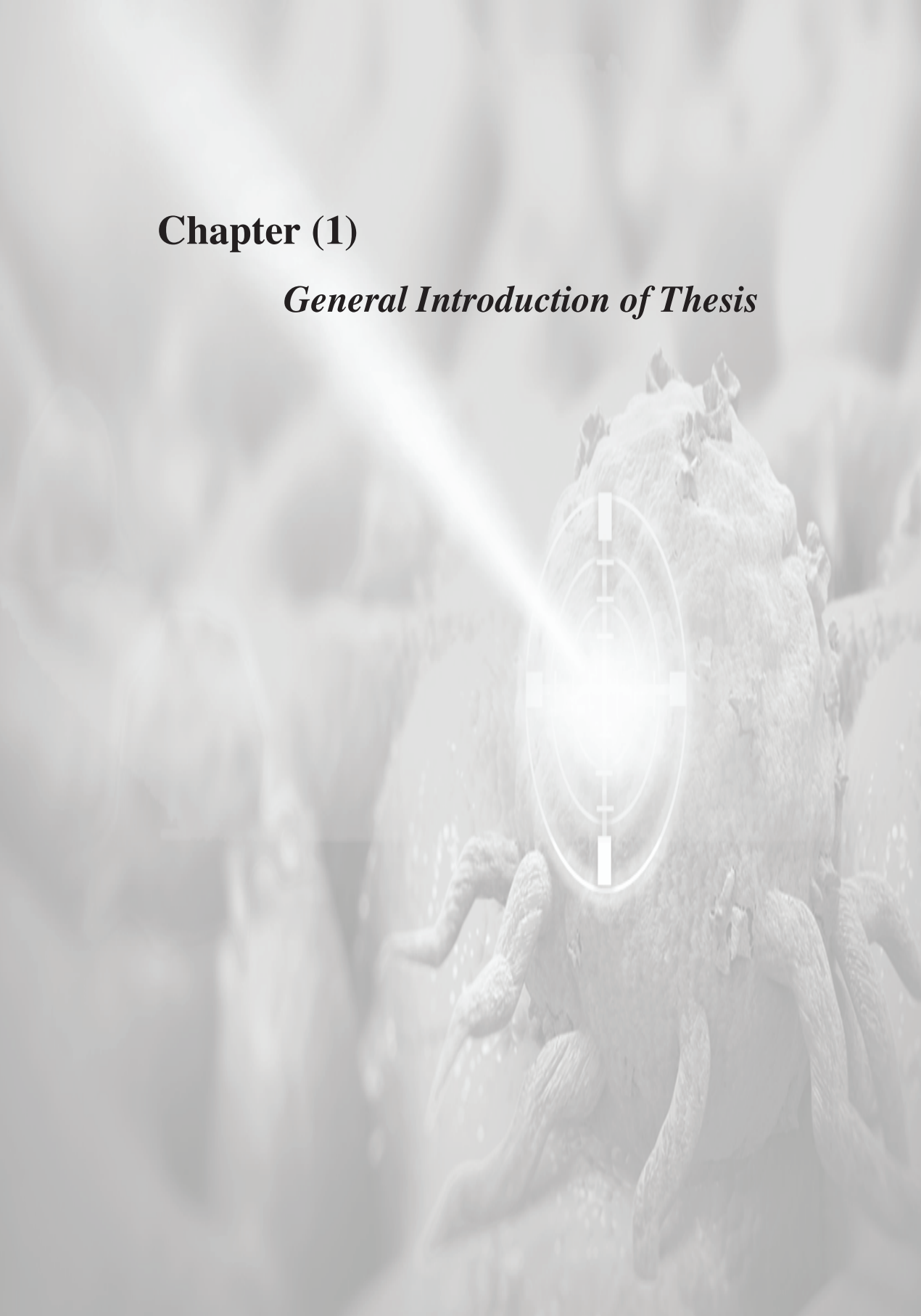
Chapter (1)

General Introduction of Thesis



Introduction to the thesis

The use of chemical agents in cancer therapy was first initiated in 1946 when a patient with non- Hodgkin's lymphoma was injected with mustine (anti-neoplastic alkylating agent) (1). Since then the use of chemotherapeutic agents has passed through multiple phases in order to improve the effectiveness and overcome its drawbacks such as poor solubility and non-specific distribution, which kills the tumor tissue as well as healthy ones and leads to severe toxicity (2, 3).

One of the approaches to address these pitfalls was by the implementation of a "magic bullet" notion, where the drug molecules are targeted via a carrier to the cancer cell, thus lowering unwanted toxicity (4, 5). A comprehensive understanding of the cancer cell complexity and biology along with their surrounding microenvironment is pivotal to design a cancer targeted drug "magic bullet" with high efficacy (6, 7). Targeted cancer therapy ensures proper biodistribution of the chemotherapeutic agent, thus selective killing of the cancer cells (8). The general concept of targeted cancer therapy is to employ a molecule, known to bind exclusively to a cancer cell specific marker (this could be a protein, gene or cancer related microenvironment), conjugated to another molecule (either a cytotoxic drug or a therapeutic enzyme) that will be delivered specifically to the cancer cell site (9, 10). In case of a therapeutic enzyme, it is directed to the tumor cells to activate the injected non-active prodrug to a highly toxic drug at the vicinity of tumor (11). The targeting molecules can be antibodies (or a fragment of antibody), ligands, vitamins, cells (some immune cells and stem cells), viruses and bacteria. They are

utilized in a variety of ways to deliver cytotoxic drugs, genes or therapeutic enzymes to tumor site thus induce cancer cell death (12-15).

Antibody Directed Enzyme Pro-Drug Therapy (ADEPT) (16-20) is a therapy that has been successfully used in animal tumor models of human choriocarcinoma, as well as colorectal and breast carcinoma. Glucarpidase (also known as carboxypeptidase G2, CPG2) is the most widely used enzyme in the ADEPT-based clinical trials (21). Glucarpidase has proved particularly useful in ADEPT, in which it accumulates at the site of a tumor via a tumor-specific antibody, after that it converts a prodrug into an active drug (21, 22). Due to the fact that ADEPT has to be used in multicycles, patients often raise antibodies which undermine the effectiveness of the treatment.

The current thesis covers work in relation to the ligand-targeted therapy, which is generally reviewed in **Chapter 2**. The major two limitations of the chemotherapeutic agent are their stability, and immunogenicity, for which several ways can be used to control these limitations (23). Carboxypeptidase G2, CPG2 has been used in targeted cancer therapy (ADEPT), this strategy suffers from some pitfalls such as immunogenicity towards the CPG2. In **Chapter 3** we employed two well-known methods (PEGylation and conjugation with human serum albumin "HSA") to enhance the stability of the therapeutic enzyme CPG2 along with reducing its immunogenicity (24, 25). In **Chapter 4** we demonstrated generation of a novel CPG2 conjugate that can be utilized in targeted cancer therapy for tumors highly expressing a cancer marker aminopeptidase N "APN". Furthermore, the success of PEGylation and conjugation with HSA in improving CPG2 therapeutic properties (26) compelled us to apply it on the newly developed CPG2

conjugates, **Chapter 5** presents the production and the in vitro study of the PEGylated CPG2 conjugates properties.

Production of a "biobetter" therapeutic enzyme to be utilized in targeted cancer therapy

To restrict the action of a cytotoxic substance to the cancer site only, an inactive form of the drug (prodrug) has been used which will be activated by an enzymatic action at the vicinity of tumor cells. Carboxypeptidase G2 (CPG2) is an exopeptidase extensively utilized in association of benzoic mustard prodrugs for targeted cancer therapy (ADEPT or GDEPT) (27, 28). Although the enzyme has reached clinical trials, due to CPG2 non-human origin, this triggered an immune response upon repeated administration cycles for treatment (21). Furthermore, the degradation effect of blood proteases was another limitation confronting the use of CPG2 as therapeutic enzyme. Conjugation/fusion of a biomolecule (such as enzymes) with other moieties such as polyethylene glycol (PEGylation), human serum albumin (HSA) are known to induce changes (structural and functional) and enhance their therapeutic properties (24, 25). Thus, in **Chapter 3** we exploited such techniques and investigated their effect with CPG2 therapeutic features. The produced new CPG2 variants (PEGylated CPG2 and HSA-fused CPG2) were tested for their stability in human serum under physiological conditions and their potential to induce an immunogenic response (enhance T cell proliferation) in human peripheral blood mononuclear cells (PBMCs). Furthermore, we were interested to get insight about any structural changes that could probably occur using Circular Dichroism (CD) spectroscopy. CD is an ideal method for rapid determination of protein secondary structure, to

study structural changes resulting from mutations, heat or denaturants on proteins (29). It is considered as a special type of absorption spectroscopy, where the optical transitions of polypeptide backbone amides are split into various transitions resulting in specific CD spectra for certain structural compositing (30). For example, proteins with α- helical structure give negative and positive bands at 222 to 208nm and 193nm respectively. Whereas, β-pleated sheets (β-helices) show their CD spectrum negative bands at 218nm and positive bands at 195nm (31, 32).

Additionally, we investigated CPG2 kinetic activity following PEGylation and HSA-fusion in comparison with the free wild type CPG2. The applied methods (PEGylation and fusion with HSA) Produced CPG2 derivatives (PEGylated-CPG2 and HAS-CPG2) with lower immunogenicity and higher stability and with extended half-life than the free CPG2.

Design and generation of cancer cell specific Enzyme-peptide conjugates for targeted cancer therapy

Studies in cancer biology revealed a variety of aberrant overexpression of markers (targets) on cancer cells. Such cancer specific targets have been utilized in cancer targeted therapy by developing drug conjugates harboring ligands specific to cancer cell receptors (33, 34). The targeting ligands are known to vary in their size, chemical structure and availability. The use of peptides as targeting ligands and its conjugation with the enzyme is much easier to perform than the conjugation of the whole cancer specific antibody. Due to its small size it might be easier for the penetration of the therapeutic complex and delivery to cancer cells (35) and cheaper to produce than the antibodies. The tripeptide, NGR (asparagine-glycine-arginine) and its cyclic

derivative (CNGRC) have been recently broadly used in targeting Aminopeptidase N (APN) highly expressed by tumor cells (36, 37). In **Chapter 4** we designed and successfully produced novel CNGRC-CPG2 conjugates, single (at the N terminal) and double (at the N, C terminals). The tri-peptide (NGR) binds to APN expressed on cancer cells, thus the binding capability of the generated conjugates to highly expressing APN cancer cells was investigated in vitro. We also carried out CPG2 catalytic activity of the conjugates. To investigate the therapeutic potential of the produced CPG2 conjugates, we investigated their stability and immunogenicity, in addition to the cytotoxic effect of prodrug (ZD2767P) in association with the CPG2 conjugates using APN expressing cancer cell lines. The findings indicated the positive effect of CPG2 conjugation with CNGRC on its potential therapeutic efficacy. Moreover, the double fused conjugate demonstrated improved properties compared with the single fused one.

Our previous studies show that CPG2 PEGylation resulted in a "biobetter" CPG2 variant (PEGylated CPG2) (26). To complete the production of long acting CPG2 conjugates we embarked on the task of PEGylation of the new CPG2 conjugates (**Chapter 5**). Both single and double fused CPG2 conjugates were successfully PEGylated and the resulting PEGylated CPG2 conjugates and we carried out molecular characterization of the new products. We evaluated their stability and ex-vivo immunogenicity of the PEGylated single and double fused CPG2.

In **Chapters 6, 7** and **8,** a summary of the thesis content is provided along with general conclusion and future perspectives.

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